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Molecular basis for group B β-hemolytic streptococcal disease

CARL G. HELLERQVIST, HAKAN SUNDELL, AND PETER GETTINS

Department of Biochemistry and Pediatrics, Vanderbilt University School of Medicine, Nashville, TN 37232

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ABSTRACT **Group B β-hemolytic *Streptococcus* (GBS) is a major pathogen affecting newborns. We have investigated the molecular mechanism underlying the respiratory distress induced in sheep after intravenous injection of a toxin produced by this organism. The pathophysiological response is characterized by pulmonary hypertension, followed by granulocytopenia and increased pulmonary vascular permeability to protein. ^{31}P NMR studies of GBS toxin and model components before and after reductive alkaline hydrolysis demonstrated that phosphodiester residues are an integral part of the GBS toxin. Reductive alkaline treatment cleaves phosphate esters from secondary and primary alcohols and renders GBS toxin nontoxic in the sheep model and inactive as a mediator of elastase release *in vitro* from isolated human granulocytes. We propose that the interaction of cellular receptors with mannosyl phosphodiester groups plays an essential role in the pathophysiological response to GBS toxin.**

Infection with group B β-hemolytic *Streptococcus* (GBS) is associated with high morbidity and mortality rates in newborn infants (1). GBS pneumonia, "early-onset disease," presents on the day of birth with signs of sepsis, granulocytopenia, and respiratory distress, characterized by pulmonary hypertension and proteinaceous pulmonary edema. Early-onset disease has been compared to Gram-negative endotoxin shock (2, 3), suggesting the involvement of extracellular toxin(s) (4, 5).

We have described (6) an extensive bacterial and media component fractionation procedure, which led to the isolation of a polysaccharide toxin from the culture media of a type III group B *Streptococcus*. This toxin, when infused into sheep, caused pulmonary hypertension, increased vascular pulmonary permeability, changes in lung mechanics, and granulocytopenia with accumulation of granulocytes in the lung (7–9).

Structural analysis of the polysaccharide component of GBS toxin has indicated a repeating unit of nine mannose residues. The determination of the structural feature of the polysaccharide essential for biological activity has been the subject of the highest priority. The data presented herein suggest that a phosphodiester residue linking a mannosyl residue to the mannan is required for pathophysiological activity of GBS toxin. Its presence and identification are based on NMR studies. Its role in inducing respiratory distress in the sheep and release of elastase from granulocytes *in vitro* is based on physiological data obtained with the native GBS toxin before and after reductive alkaline hydrolysis.

MATERIALS AND METHODS

Bacterial Cultures. GBS type III cultures were initiated from the original stock inoculum (6) and stored at −70°C by incubation in Todd–Hewitt broth (THB) for 9 hr at 37°C. Sheep blood agar (SBA) plates were inoculated and incubated overnight. Several colonies were picked and grown in THB overnight, and these cultures were used for inoculation of mice.

***In Vivo* Passage of GBS.** Mice were inoculated subcutaneously with 0.3×10^9 bacteria and were sacrificed when they were seriously ill. The spleen was excised, and a SBA plate was inoculated using the intact spleen, which was then placed in 10 ml of THB. Cultures were taken from the SBA plate and used for inoculation of eosin methylene blue (EMB) plates, trypticase soy agar (TSA) plates, and Sabouraud dextrose agar (SDA) plates. After an overnight incubation at 37°C, the broth culture was used as described above for inoculation of a second mouse, provided the SBA, EMB, TSA, and SDA plates demonstrated that the cultures were homogeneous and free of any microbial contamination. One each of the TSA and SDA plates were also incubated at 24°C and checked after 48 hr. In case of any contamination, several pure colonies of GBS were picked and inoculated overnight in THB and used as an inoculum for the next mouse. Bacteria obtained after five passages through mice were used for GBS toxin production.

Quality Controls. In addition to subjecting our bacterial cultures to the above described procedures, each fraction obtained during the purification procedures was inoculated under the same conditions as above to allow certification of purity (i.e., absence of any microbial contamination) (6).

Purification of GBS Toxin. The procedures described previously were used with minor modifications (6). Autoclaved culture supernatants were made 70% in ethanol. The precipitate was subjected to phenol/water extraction (10), and the water phase was dialyzed against water and applied to a DE 52 column (Whatman) developed in water. The material eluted at 0.15–0.25 M NaCl was pooled, dialyzed, lyophilized, and subjected to gel filtration on a Sephacel S-300 column (5 × 100 cm) (Pharmacia). The column was developed with 0.15 M NaCl buffered at pH 6.5 with 0.01 M NaOAc. GBS toxin was collected in the void-volume peak.

Sugar Analysis. Material (200 μg) was dissolved in 400 μl of 2 M H_2SO_4 in a 5-ml ampule. Glacial acetic acid (3.60 ml) was added, and the material was heated for 9 hr at 100°C (11). Acetic acid was removed by repeated evaporations with added water, and the remaining sulphuric acid was neutralized with $BaCO_3$. Monomers were converted to the corresponding alditol acetates, which were purified by charcoal filtration in ethyl acetate and analyzed by GLC/MS (12).

Methylation Analysis. The methods pioneered in 1968 (12) were used with a slight modification devised to assure complete hydrolysis of glycosidic linkages involving glycosaminidic (11) as well as glycuronidic linkages (13). Fully methylated material, purified by dialysis, was dissolved in glacial acetic acid (3.60 ml), and 400 μl of 2 M H_2SO_4 was added. After 9 hr at 100°C, the resulting monomers were converted to alditol acetates and analyzed by GLC/MS (12) with a OV 225 fused silica column (Quadrex, New Haven, CT) on a Sigma 1 gas chromatograph (Perkin–Elmer) and a

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Abbreviation: GBS, group B β-hemolytic *Streptococcus*.

flame-ionization detector for quantitative analysis and a combined GLC/MS (Rebair model R1010C, Paris) for qualitative determinations.

Reductive Alkaline Hydrolysis. GBS toxin (10 mg) was subjected to reductive alkaline hydrolysis (14) at 80°C for 1 hr with 0.1 M NaOH made 1 M in $NaBH_4$. Borate was removed by repeated evaporations with methanol (14). The resulting material was fractionated on Sephadex G-10, and the polymeric (designated Red Alk toxin) and monomeric materials were collected and subjected to structural analysis.

NMR Measurements. ^{31}P NMR spectra were recorded at 162 MHz on a Bruker AM400 narrow-bore spectrometer; 2- to 5-mg samples in 99.8% ^2H_2O were used. ^{31}P-selective probe was used for the measurements. Unless stated otherwise, 2-W broad-band protein decoupling was used. Other parameters were typical: sweep width of 4000 Hz, pulse width of 5 μs (30°C), and repetition rate of 0.5 s^{-1}.

^1H NMR spectra were recorded at 400 MHz. One-dimensional spectra were acquired over 2.7 S with an 8 μs (60°) pulse, and a sweep width of 2500 Hz. The two-dimensional homonuclear chemical-shift correlation data set was collected by using a 90°–τ–45° acquire-pulse sequence (15) with 2000 data points in the F_2 dimension and 128 spectra in the F_1 dimension zero-filled to 1028 data points prior to Fourier transformation. A shifted sine-bell weighting (16) with a phase shift of $\pi/5$ was used.

Sheep Model. Yearling sheep (35–45 kg) were prepared for chronic lung-lymph collection as described (7). Through bilateral thoracotomies, catheters were placed directly into the main pulmonary artery, the left atrium, and the efferent duct from the caudal mediastinal lymph node. The tail of the node and systemic contributaries were ligated. All experiments were done with unanesthetized unrestrained sheep, standing in a cage (7). Vascular pressures were measured continuously with Statham pressure transducers (model P23Gb; Gould–Statham Instruments, Hato Ray, PR) and recorded on a recording system (model 88005, Hewlett–Packard). Rectal temperature was monitored with a telethermometer (Yellow Springs Instrument). Leukocytes were counted with a model 2BI Coulter Counter.

GBS toxin or modified GBS toxin was dissolved in 80 ml of saline (0.9% NaCl) and infused over 15 min into the pulmonary artery after a stable baseline had been established during 2 hr. Data were collected every 15 min initially and every 30 min thereafter for 5 hr.

Preparation of Human Granulocytes. Human blood (60 ml) was collected in 6 ml of acid citrate dextrose. Seven milliliters of blood was layered on 3.5 ml of mono-poly resolving medium (Flow Laboratories) and centrifuged at 1000 × *g* for 30 min. Granulocyte layers were collected from several tubes, pooled, and washed twice by centrifugation in Hepes-buffered Hanks' solution (pH 7.2) supplemented with the essential and nonessential amino acids of minimal essential medium (medium A).

Elastase Assays. Human granulocytes were incubated for 18 hr at 37°C with variable amounts of microbial polysaccharides. Aliquots (100 μl) of granulocyte suspensions containing 1.6×10^8 granulocytes were mixed in microcentrifuge tubes with 100 μl of polysaccharide solutions in medium A for a final concentration of 0–100 μg per ml. After completion of the incubation on a rocking platform (1 rock per 5 sec), the granulocytes were pelleted, and the supernatant was transferred to microcentrifuge tubes containing 2 mg of washed orcein. Tris buffer (0.15 M; 100 μl) at pH 10.2 was added to reach a final pH of 8.6, and the incubations were continued on the rocking platform at 37°C for 2 hr. Elastase was inactivated by the addition of 200 μl of 0.5 M sodium phosphate (pH 6), the tubes were centrifuged, and the supernatant was read at 590 nm.

Lung lymph elastase levels were determined by incubating on a rocking platform (2 hr at 37°C) 200 μl of lung lumph, obtained from sheep before and after infusion of 2 mg of GBS toxin, with 2 mg of washed elastin beads suspended in 200 μl of 1 M Tris (pH 10.0) for a final pH of 8.6. The incubations were terminated as above, and elastase levels were determined spectrophotometrically as above.

RESULTS

Tentative Carbohydrate Structure. Quantitative and qualitative sugar analysis of GBS toxin by GLC/MS gave only a mannitol derivative and demonstrated the absence of heptose and 2-amino-2-deoxyglucose derivatives, which would have been indicative of Gram-negative lipopolysaccharide contamination (6, 12).

Methylation analysis gave the derivatives shown in Table 1. Stoichiometrically, the 2,3,4,6-tetra-*O*-methyl derivatives and the 3,4-di-*O*-methyl derivatives are indicative of a triple-branched repeating unit of nine mannose residues.

In addition to GBS toxin, the medium fraction yields a GBS mannan with no significant charge but with a similar if not identical carbohydrate structure as judged by ^1H NMR and methylation analysis (unpublished data).

Effect of Reductive Alkaline Hydrolysis. Red Alk toxin (500 μg) was subjected to methylation analysis. The resulting methyl ethers presented in Table 1 indicate an identical stucture to the native GBS toxin. The absence of detectable amounts of mannitol derivatives methylated at C-1 and C-5 demonstrates the polymeric nature of the material. This material, Red Alk toxin and native GBS toxin, was used for NMR and for *in vivo* and *in vitro* studies described below.

Analysis of the material included on the Sephadex G-10 column yielded only mannitol as analyzed by GLC.

^{31}P NMR Spectrum of GBS Toxins. The ^{31}P NMR spectrum of GBS toxin (Fig. 1, spectrum A) has a major peak at −1.7 ppm (90% of the total phosphorus present) and two minor peaks at 0.2 ppm and 0.8 ppm. In all samples of GBS toxin examined, the large upfield resonance is always present, whereas the smaller resonances are variable. The resonance at −1.7 ppm corresponds in chemical shift to an acyclic phosphodiester (17), while the smaller resonances are more likely to be phosphomonoesters. The resonances at 0.2 and 0.8 ppm move downfield to 4.8 ppm, while the peak at −1.7 ppm is unaffected by addition of NaOH (Fig. 1, spectrum B). This behavior is as expected for mono- and diesters, respectively. Phosphomonoesters ionize from the monoanionic to dianionic forms in the pH range 5–7 with a change in chemical shift of ≈4 ppm downfield. In contrast, phosphodiesters have a single ionization with a pK_a of about 1.5 and, therefore, are completely ionized at all pH values > 3.

To this sample was added 10 M NaB^2H_4 in 1 M NaO^2H to give a final NaB^2H_4 concentration of 1 M. ^{31}P spectra were then collected every 5 min for 50 min and showed a progressive loss of intensity at −1.7 ppm and concomitant increase in intensity at 4.8 ppm. The downfield resonance (Fig. 2,

Table 1. Methylation analysis of original and Red Alk GBS toxin

Oxidized methyl ether derivatives of mannitol	Molar ratios	
	Original GBS	Red Alk toxin
2,3,4,6-Tetra-*O*-Me	33	33*
3,4,6-Tri-*O*-Me	13	15
2,4,6-Tri-*O*-Me	10	10
2,3,4,-Tri-*O*-Me	10	10
3,4-Di-*O*-Me	33	37

*Normalized to the amount of 2,3,4,6-tetra-*O*-Me in the original GBS.

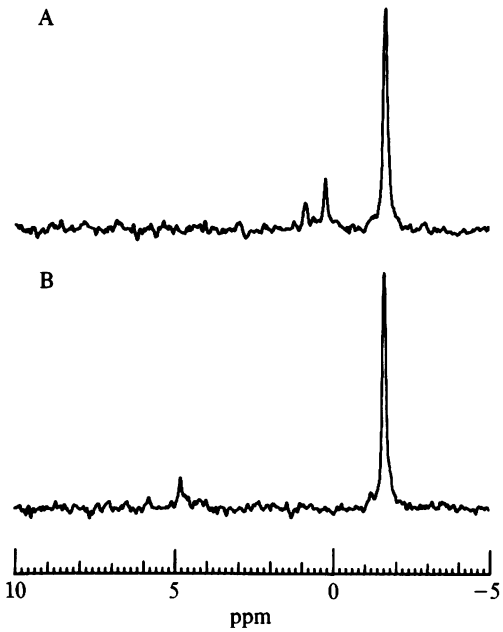

FIG. 1. ^{31}P NMR spectra at 162 MHz of toxin at pH 5.0 (spectrum A) and after addition of NaOH to make the solution alkaline (spectrum B). Each spectrum represents the average of 2048 scans.

spectrum B) is composed of two resonances with slightly different chemical shifts. The linewidths of these resonances are estimated to be 15 Hz. To demonstrate that the new resonances were from phosphomonoesters rather than inorganic phosphate, a spectrum was recorded of the NaB^2H_4-treated toxin to which inorganic phosphate was added. The phosphate resonated 2.3 ppm upfield from the toxin reaction product resonance (spectrum not shown).

^1H and ^{13}C NMR Spectra of Toxin. The anomeric region of the ^1H NMR spectrum of GBS toxin (Fig. 3, spectrum A) has resonances at 5.055, 5.098, 5.118, 5.150, 5.170, 5.300, 5.420, 5.443, and 5.460 ppm. The important features with regard to the phosphodiester present are the three peaks at 5.460, 5.444, and 5.420 ppm. Reaction of the toxin with NaB^2H_4 in NaO^2H, which cleaves the phosphodiester to give a phosphomonoester and presumably a free sugar, resulted in a complete loss of these resonances, whereas the gross features

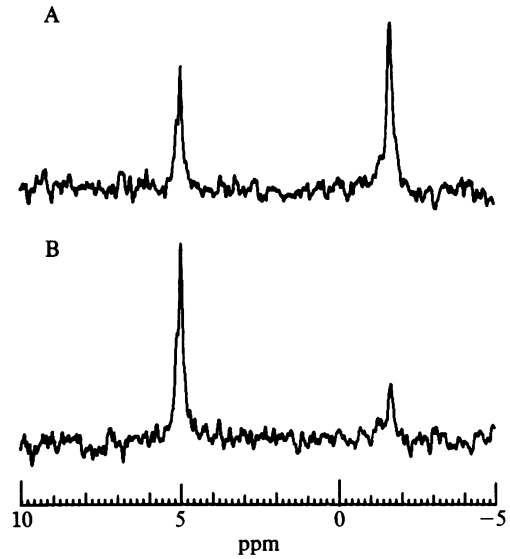

FIG. 2. ^{31}P NMR spectra at 162 MHz of GBS toxin at 20 min (A) and 50 min (B) after the addition of NaB^2H_4 to a concentration of 1 M. Spectrum B represents 20 min of averaging centered at $t = 50$ min.

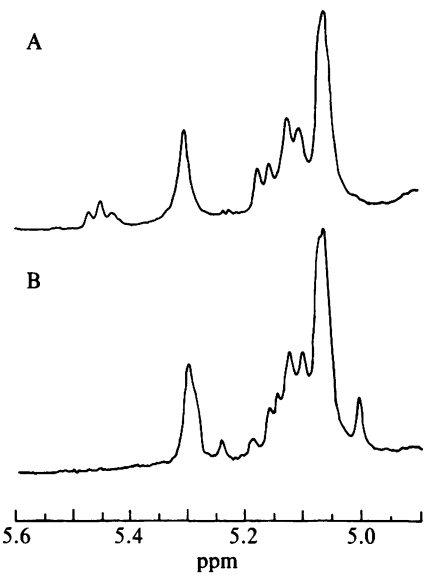

FIG. 3. ^1H NMR spectrum at 400 MHz of GBS toxin (spectrum A) and GBS toxin after treatment with NaB^2H_4 in NaOH (Red Alk toxin) and subsequent removal of borate (spectrum B). Spectra were recorded at 303 K and are the average of 2048 scans. Only the region of the spectrum in which the anomeric protons resonate is shown.

of the remainder of the anomeric region spectrum were not dramatically altered, with the exception of a new small resonance at 5.001 ppm (Fig. 3, spectrum B). Upon dialysis of the reaction products and lyophilization of the dialysate, only a simple mannitol-type substance was found, which gave a ^1H NMR spectrum with resonances between 3.5 and 4.1 ppm.

In the two-dimensional chemical shift correlation plot (not shown), there are two cross peaks corresponding to the three peaks centered at 5.445 ppm. Connectivities are between a resonance at 5.430 ppm and one at 4.139 ppm and between a resonance at 5.452 ppm and one at 4.017 ppm. Thus, the three peaks at 5.420, 5.443, and 5.460 ppm seem to consist of two overlapping doublets rather than three separate resonances with resolvable 8-Hz coupling. Irradiation at 4.017 ppm or 4.139 ppm did not collapse this coupling, and so it must be concluded that the coupling is heteronuclear, presumably to ^{31}P. A ^1H spectrum of α-D-mannopyranosyl 1-phosphate (not shown) gave an anomeric proton resonance at 5.404 ppm with 8-Hz coupling to phosphorus and 2.1-Hz coupling to the C-2 proton at 3.959 ppm.

The anomeric region of the ^{13}C NMR spectrum of the unreacted GBS toxin (not shown) has a major resonance at 102.96 ppm, resonances of ≈30% and ≈40% of this intensity at 101.38 and 99.07 ppm, and much smaller peaks (10% intensity) at 103.85 and 97.03 ppm. The three major resonances are unaffected by treatment with NaB^2H_4/NaO^2H.

Quantification of Phosphodiester. The concentration of phosphodiester present in the GBS toxin was estimated from a ^{31}P NMR spectrum using a coxial α-D-mannopyranosyl 1-phosphate external standard. A concentration of 3.3 mM phosphodiester was determined. Given that GBS toxin is purely mannose (6), this corresponds to one phosphodiester per 19 mannose residues.

An independent estimate obtained from the ^1H NMR spectrum, by using the relative peak areas of the three-peak group at 5.440 ppm and the remainder of the anomeric proton region, gives a value of 1 phosphodiester per 18 mannose residues.

Sheep Studies. Table 2 summarizes changes in the response in three sheep given intravenous injections of GBS toxin. In the three sheep, increases in pulmonary artery pressure varied from 125% to 205% and increases in rectal temperature

Table 2. Physiologic response in three sheep to GBS toxin

Sheep	Response*	Pulmonary artery pressure, torr†	Rectal temp, °C	Leukocytes per mm³
28–86	Baseline	19	39.4	9,934
	Phase 1	58	39.7	4.233
	Phase 2	25	41.8	4.274
21–86	Baseline	19	39.7	9,692
	Phase 1	48	39.7	3.361
	Phase 2	25	41.6	1,887
31–86	Baseline	19	40	10,507
	Phase 1	43	40.5	2,159
	Phase 2	25	41.7	1,866

Temp, temperature.
*Phase 1, 0.5–1 hr after GBS toxin; phase 2, 3.5–5 hr after GBS toxin.
†1 torr = 133 Pa.

varied from 1.7°C to 2.4°C. Leukocyte counts in the blood decreased by 58–82%.

When Red Alk toxin was infused, there were no changes seen in any of the monitored variables. In addition, 2 mg of a GBS mannan isolated from the culture medium, having a similar if not identical carbohydrate structure but with a very small number of phosphodiester residues (1 per 20 repeating units), gave an increase in pulmonary artery pressure from 21 to a peak of 28 and a dip in leukocytes from 7100 to 4500 when infused in the sheep. GBS mannan with no detectable charged groups in three separate infusions in sheep gave no physiologic response.

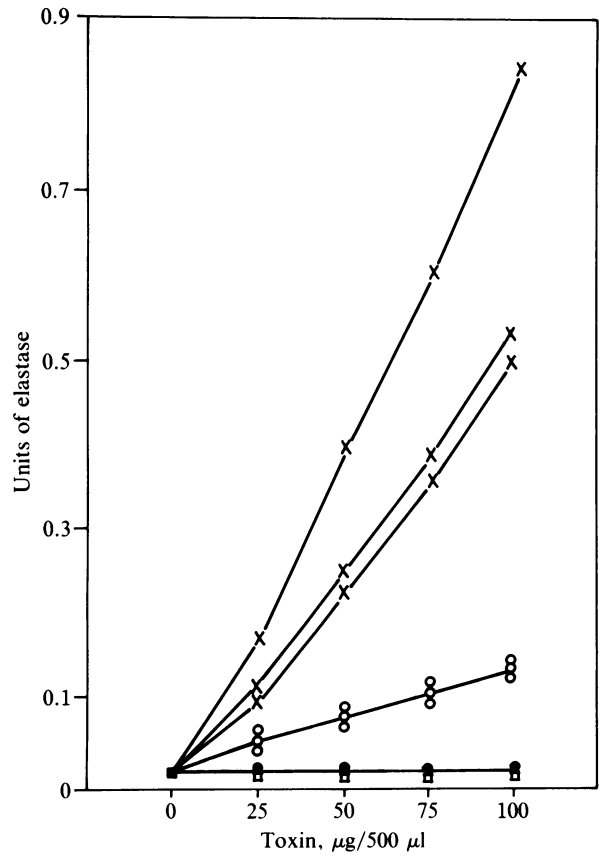

FIG. 4. Human granulocytes (1.6×10^8) were incubated with GBS toxin (×), Red Alk toxin (○), and control polysaccharides GBS mannan (●) and dextran (□) at the indicated amounts in a total volume of 200 μl of medium A for 16 hr at 37°C. Elastase released was determined by incubating the supernatants with 2 mg of orcein-impregnated elastin beads for 2 hr at 37°C and reading the OD at 590 nm. Calibration curves were prepared by using 2 mg of elastin beads and commercial enzymes of defined activity.

Lymph collected from a sheep over the duration of the response to GBS toxin infusion when assayed for elastase showed an increase from a baseline value of 0.08 unit (see the legend to Fig. 4) to 0.15 at the peak of the pulmonary pressure increase (phase 1), at which time lung lymph flow had increased from 3.0 to 9.0 ml per 15 min. The peak in elastolytic activity occurred 3 hr after infusion (phase 2) with 0.37 unit of elastase per 200 μl of lymph, when lymph flow was 8 ml per 15 min.

***In Vitro* Assay of Elastase.** To substantiate the role of the phosphodiester residues in the response by the granulocytes, *in vitro* granulocytes were incubated with GBS toxin and Red Alk toxin. Fig. 4 depicts the results from three separate determinations; as seen, the Red Alk toxin failed to elicit the effect on the granulocytes seen with the native GBS toxin. With no detectable phosphodiester residues present, the slight elevation seen over background with Red Alk toxin when compared with dextran and mannan may indicate a partial cell lysis or elastase release induced by residual trace amounts of borate esters on the Red Alk toxin.

^{32}P-Labeled GBS Toxin. GBS were incubated with 5 mCi (1 Ci = 37 GBq) of [^{32}P]phosphate under standard culturing conditions, and GBS toxin was isolated by the procedures outlined above. GBS toxin (5.4 mg) was infused in a 12-day-old lamb, whose response was monitored. Pulmonary artery pressure increased from 19 to 44 torr. Arterial oxygen tension decreased from 100 to 77 torr, and circulating leukocytes decreased to 1500 from 8000. After 1 hr the animal was sacrificed. All major organs were then removed, weighed, and sampled, and the samples were lyophilized. Table 3 shows that 50% of the total cpm in the animal had accumulated in the lungs and liver, which together constitute <5% of the total weight of the animal.

DISCUSSION

The molecular mechanism of GBS-induced respiratory distress in neonates and our experimental sheep model has been the subject of studies aimed at the prevention of the often fatal pulmonary damage that follows GBS infection in neonates.

We have demonstrated previously in sheep that pathophysiological changes mimicking those of GBS infection in neonates can be induced by infusion of a polysaccharide component (GBS toxin) isolatable from the bacterial culture media (6–9). The data presented here suggest that GBS toxin

Table 3. Dispersion of ^{32}P-labeled GBS toxin in a 12-day-old lamb

	Total cpm*	Error
Right lung unwashed	93,500	1%
Left lung after airway lavage	52,050	1%
Liver	25,050	3%
Kidney	6,060†	3%
Muscle	300 per 15 g	7%
Spleen	4,800	3%
Cerebrum	3,880	5%
Brain stem	350	5%
Lymph gland	350	5%
Heart	3,150	5%
Thymus	600	5%
Lavage supernatant	5,000	5%
Lavage pellet	0	
Erythrocytes	0	
Plasma at 30 min after infusion‡	48,000	
Plasma at time of sacrifice‡	30,000	10%

*Total cpm infused = 384,084 cpm.
†3030 × 2 = 6060.
‡Estimate assuming total vol of 560 ml.

is a mannan with a nine-mannose-residue repeating unit and that the active site on the polysaccharide is a phosphodiester-linked mannosyl residue. Evidence for this was obtained by ^{31}P NMR studies of GBS toxin isolated from cultures grown after five consecutive *in vivo* passages in mice and from comparative *in vitro* and *in vivo* studies utilizing GBS toxin and GBS toxin subjected to reductive alkaline hydrolysis.

From its chemical shift and failure to titrate downfield in alkaline, the principal resonance in the ^{31}P NMR spectrum of the GBS toxin clearly arises from phosphodiesters. Reaction of GBS toxin with NaB^2H_4/NaO^2H yields a mannan and phosphomonoesters that are subsequently reduced to mannitol. The disappearance of the proton resonances at 5.440 ppm suggests that the mannose residue(s) cleaved from the phosphate is linked to C-1. This is supported by the existence of 8-Hz heteronuclear coupling in this group of anomeric protons, which must arise from H-C-O-P three-bond coupling. The magnitude of the coupling and also the ^1H chemical shifts are similar to those seen in mannose 1-phosphate. The site of linkage to the toxin core is not identified in this study.

Two ^1H resonances from mannose residues involved in phosphodiester linkages are seen with chemical shifts of 5.452 and 5.430 ppm. Although the corresponding ^{31}P resonances are not resolvable from one another upon cleavage of the phosphodiester, the product phosphomonoesters reflect the existence of two distinct sites of phosphate linkage in the asymmetry of the ^{31}P resonance. The apparent existence of two distinct sites of phosphorylation with approximately equal frequency means that each type of phosphodiester occurs once every four repeating units or once per 36 mannose residues.

The minimal changes in the ^1H and ^{13}C spectra, apart from those accounted for by the removal of the phosphodiester residues, indicate that no other structural changes are caused by the reductive alkaline treatment.

^{31}P NMR studies on the Red Alk toxin, which methylation analysis showed to be of high molecular weight, revealed that no phosphate groups were present.

The physiological role of the mannosyl phosphodiester residues is indicated by the observations that, whereas native GBS toxin when infused in sheep induces increased pulmonary arterial pressure, granulocytopenia, fever, and enhanced pulmonary vascular permeability, Red Alk toxin has no measurable effect, although the polysaccharide structure appears to be virtually identical. This conclusion is further corroborated by the observations that (*i*) GBS toxin obtained from an initial culture prior to mouse passages with <10% of the phosphodiester residues had a markedly reduced potency when infused in the sheep, and (*ii*) GBS mannan with no detectable charged groups had no physiologic effect in the sheep.

In vitro the mannosyl phosphodiester residues appear essential for the release of elastase from human granulocytes. Red Alk toxin is less effective, and GBS mannan and dextran are ineffective as release mediators of elastase. If the release were due to cell lysis, then it would be an induction by GBS toxin.

Granulocytes and macrophages are the major carriers of elastase (18); thus, the granulocytes adhering to the lung endothelium (9) *in vivo* are prime candidates as a source for the elevated lymph elastase level seen in response to GBS toxin.

Thus, the data presented in this study suggest that mannosyl phosphodiester residues are involved in the pathophysiology of GBS disease. The response *in vitro* of granulocytes to GBS toxin and the accumulation of labeled GBS toxin in the liver and lung suggests that GBS toxin elicits its physiologic effects through specific interactions with cellular receptors on target cells within these organs. The formation of these structural features appears to be correlated with the number of *in vivo* passages in mice, which in turn seems to be correlated with the appearance of a 20-kilobase plasmid.

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